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Contract No. DA-18-108-405-CML-120

ANNUAL COMPREHENSIVE REPORT

1 January 1961 - 31 December 1961

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December 1961

PART I

TACHYPHYLAXIS AND TOLERANCE TO ANTICHOLINESTERASE DRUGS IN MAN
with Michael P. McQuillen, M.D.INTRODUCTION

A patient was recently referred to us because of alleged myasthenia gravis. The only clinical evidence suggesting myasthenia in this patient was the fact that she tolerated massive doses of anticholinesterase medication without untoward effect (it is generally considered that only patients with myasthenia gravis can tolerate such massive doses of anticholinesterase medication).

Review of our clinical material, prompted by this patient, revealed four other similar patients. These cases are summarized in Table 1. Each had complaints of weakness and asthenia which were not typical of myasthenia. Muscarinic and nicotinic effects occur after a single dose of 15 mg neostigmine, 60 mg pyridostigmine, or 5 mg ambenonium in normal subjects. With three times that dose serious effects are invariably seen. Since these patients did not show these effects, each was considered to have myasthenia on the basis of this tolerance to large doses of anticholinesterase drugs.

While the occurrence of such remarkable tolerance is of interest, its mechanism is of potential importance in counteracting the effects of anticholinesterase chemical warfare agents. Clinical investigation of this patient will be described in this section while other investigations of this problem will be described in subsequent sections.

CLINICAL ABSTRACT

G.K. (JHH #99 31 31), a 60 year old white female, was admitted for the first time 31 May 1961 for evaluation of generalized, although largely proximal, muscle weakness of five years' duration.

She had been well until 1936, when the gradual onset of migratory polyarthralgias without swelling or redness was noted. In the ensuing years therapy with gold, salicylates, and cortisone was employed with little lasting relief. A mildly elevated blood glucose without glycosuria was noted in 1954, and NPH insulin 10 units was given daily. In 1959 a cataract was removed from her left eye. She was admitted later in the year to another hospital for one of several episodes of severe, left lower quadrant cramping pain. Diverticuli were demonstrated on barium enema, and it was thought one had perforated and caused a localized peritonitis.

In June of 1956 she was admitted to another hospital because of questionable pelvic and shoulder girdle atrophy with proximal limb girdle and truncal weakness that was most severe in the right arm, and most apparent in such activities as

TABLE I

PATIENT	SYMPTOMS	SIGNS	ATROPOINE e	ANTICHOLINESTERASE THERAPY	AGENT	MAXIMUM DAILY DOSE (MG)
O.E. 57WF 16 57 90	Recurrent "attacks" of crano-skeletal weakness for 39 yrs.; considerable psychiatric overlay.	No objective neuromuscular findings; normal EMG with no neuromuscular transmission defect in clinically "weak" muscles; no fasciculations.	None	Neostigmine	540	
L.W. 43WF 76 29 16	Multiple system complaints, with progressive fatigue invariably related to psychogenic background, for 10 yrs.	No objective N-M findings; no cooperation in attempting to assess any parameter of strength; no fasciculations.	Rarely	Pyridostigmine	2,160	
S.M. 20WF 97 29 61	"Lack of pep" and easy fatigability for 6 yrs.	Non-organic sensory findings; residua of 11 (mainly orthopedic) surgical procedures; no fasciculations.	None	Pyridostigmine Ambenonium	1,680 40	
E.T. 19CF 97 56 51	Onset of weakness related in time to psychological stress; eventually institutionalized for psychosis.	Generalized weakness worse after medication, but difficult to assess at any time; equivocal neostigmine test with placebo response; no fasciculations.	None	Pyridostigmine	4,580	
G.K. 60WF 99 31 31	Proximal limb-girdle weakness for 5 yrs.; nocturnal muscular episodes for 2 months.	No objective N-M findings; no N-M transmission defect in clinically "weak" muscles, fasciculations after neostigmine.	None	Pyridostigmine	1,840	

combing her hair. On repeated questioning there seemed to be some hint that this weakness would tire on repetitive activity and improve with rest. Her voice was said to become hoarse for long periods, she might have difficulty in chewing steak, and there were times when she felt as though she were choking. Physical examination disclosed pain on passive movement of almost all joints, and minimal wasting in the deltoids, flexors of the thigh, and gluteal muscles, with appropriate weakness. X-rays of the knees and lumbar and cervical spine showed osteoarthritis; on electromyography the action potentials were of low voltage and were said to show a "rapid decrease on sustained effort"; muscle biopsy was read as showing "mild atrophy of muscle, adipose and subcutaneous tissues". She was begun on a number of different medications at this time but did not improve. In December, 1956, she was given 1.5 mg neostigmine i.m. with an increase in stamina of rapid squeeze from 10 seconds to 2 minutes 30 seconds. Oral neostigmine (15 mg t.i.d.) was started, and then pyridostigmine (60 mg q.i.d.) was begun. A gradual increase in dosage to 240 mg q.3 h. over three years took place without serious, recognizable side effects. By May of 1961, however, she was having episodes of weakness, sweating, muscle twitching that would occur between 2 and 3 a.m. approximately 2 to 3 hours after her last dose of pyridostigmine.

On physical examination BP was 150/90. The patient was obese. A cataract was present on the right, with left aphakia. There were no arthritic deformities seen. A rectal stricture with external hemorrhoidal tags was present. Neurological examination was normal.

Routine laboratory studies were not remarkable. There was no evidence of diabetes.

CLINICAL INVESTIGATION

1. Strength testing. The patient's strength was evaluated 12 hours after her last dose of anticholinesterase medication (see Table 2). The intramuscular injection of atropine 0.6 mg produced a moderate increase in performance of the strength tests indicating a placebo effect. Neostigmine 1.5 mg intramuscularly produced no further change in strength. A few fasciculations were visible in the left calf.

From this neostigmine test one would conclude that the improvement in strength which was observed was attributable to placebo effect. It was of interest however, that there was no reduction in strength following 1.5 mg of neostigmine as might be anticipated in normal subjects.

2. Electromyographic investigation. During the course of the above mentioned neostigmine test the ulnar nerve was stimulated percutaneously and recordings were made of muscle action potentials from the adductor of the thumb together with its isometric tension. The myasthenic patient shows

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4.

HISTORY NO

PATIENT'S NAME

MYASTHENIA GRAVIS CLINIC

SERVICE

TABLE 2

	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT
1. DATE / TIME	2 June 61	10:10a.m.			11:40a.m.				12:36p.m.	
2. DRUG / AMT	Mestinon	240mg	Atropine	0.6mg	Neostig.	1.5mg				
3. SCHED / TAKEN		11:00p.m.			10:34a.m.				11:52p.m.	
4. EOM (deg)			Full	Full	Full	Full	Full	Full		
5. LIDS (mm) B/A	10	4	10	5	10	7	10	8	10	10
6. CHEW	dk		dk		dk		dk		dk	
7. SWALLOW	dk		dk		dk		dk		dk	
8. COUNT	dk		dk		dk		dk		dk	
9. COUGH	dk		dk		dk		dk		dk	
10. VITAL CAP										
11. ARM ABD (m:s)	0:47		0:47		1:11		1:31		1:31	
12. HAND GRIP 1/2 (kg)	84	100	104	90	100	84	50	56		
	3 4	90	82	80	76	66	40	60	44	
13. NECK FLEX (m:s)	0:57				1:46				1:08	
14. LEG FLEX (m:s)	0:19	0:32			1:03		0:56		1:08	
15. FASCICULATIONS	none				none		none		0:49	
16. SIDE EFFECTS	none				none		none		calf	
17. ATROPISE	none				none		none			
18. COMMENT	baseline strength	placebo effect			neostigmine effect					

a characteristic response in that there is reduction in the size of action potential of the second of two evoked responses. Furthermore, the administration of neostigmine to the myasthenic produces complete or partial repair of this block in neuromuscular transmission. The normal subject shows no block to neuromuscular transmission, while the administration of neostigmine is associated with repetitive firing of the muscle following a single nerve volley and reduction in size of the second of paired evoked potentials. Frequently there is potentiation of the mechanical response to a single stimulus which is attributable to the repetitive firing.

Under basal conditions, this patient's response to paired nerve stimuli was perfectly normal. There was no evidence for a myasthenic defect. Following the administration of neostigmine there was no change in the electromyographic pattern. There was no repetitive firing, decline in response to the second of paired stimuli, or potentiation of the single twitch.

In summary, the electromyographic studies showed no myasthenic defect, nor did they show the usual anticholinesterase manifestations after 1.5 mg of neostigmine.

Needle electromyograms showed some bizarre motor unit potentials and some fibrillation potentials in the left gastrocnemius. There were no other significant abnormalities.

3. Biopsy. The deltoid muscle biopsy obtained elsewhere was interpreted by Dr. William Cathey as showing moderate diffuse atrophy of muscle compatible with disuse and dermal thickening in the skin.

SUMMARY

These cases indicate clearly that tachyphylaxis or tolerance develops in man when anticholinesterase drug is given in increasing amounts over a relatively long time period. The patient presented in detail was receiving each day some 30 times the amount of anticholinesterase medication which would produce symptoms in the ordinary person. While the minimal lethal dose of these drugs is not known, it is not unreasonable to suppose that the doses taken by these patients are in that range.

Two questions arise: 1. What is the mechanism of this remarkable tolerance? 2. Can it afford prophylaxis against anticholinesterase chemical warfare agents? Follow up studies are being performed on the above described patient, and the other patients who exhibit this syndrome are being investigated. The animal experiment to be described below have been undertaken in an attempt to shed further light on this problem.

PART II

RAT WHOLE BLOOD CHOLINESTERASE METHOD

INTRODUCTION

The studies to be described in the next section required periodic estimation of rat blood cholinesterase. Since repeated examinations were required, a semi-micro method was required. Since the method was to determine the relative effect of administered anticholinesterase drug, a whole blood method was considered suitable. The method was designed to permit a large number of determinations to be done.

METHOD

1. Reagents and materials

Michel's human plasma cholinesterase buffer, pH 8
Acetylcholine iodide 3 gm per 100 ml
Heparinized transfer pipette 0.25 ml
Palo automatic pipetter 3 ml
Palo automatic pipetter 2 ml

2. Procedure

- A. Pipette 2 ml water into serology tubes with pipetter.
- B. Transfer 0.25 ml blood from tail to tube; rinse 3 times.
- C. Sample may now be sealed in refrigerator for 24 hours or frozen indefinitely.
- D. Place tube in 25° C. water bath.
- E. Add 2 ml buffer to each tube with pipetter.
- F. Add 0.5 ml ACh iodide.
- G. Measure pH of aliquot after approximately ten minutes, note time, and discard aliquot.
- H. Again measure pH of an aliquot about sixty minutes later, note time, and discard aliquot.

3. Controls

- A. Non-enzymatic ACh hydrolysis: as above but without blood (substitute water 0.25 ml).
- B. Non-ACh acid formation: as above but without ACh (substitute water 0.5 ml).

4. Calculations

Observed ChE Δ pH/hr - Control A Δ pH/hr - Control B
 Δ pH/hr = corrected ChE activity.

DISCUSSION

The method was checked for reproducibility by the serial determination of replicates. The activity of eight replicates was found to be $0.42 \pm 0.01 \Delta\text{pH}/\text{hr}$ (mean \pm S.E.M.). The linearity of the method was determined by measuring cholinesterase activities of mixtures of normal blood and blood 100% inhibited by DFP 10^{-5}M . The inhibited blood was allowed to stand for 24 hours before mixture in order to permit destruction of any excess DFP. The results are shown in Figure 1. Each point represents triplicate determinations. Substrate limitation was excluded by determining that the hydrolysis rate of normal blood did not decrease during a second hour of incubation. The addition of an hemolysin such as saponin was found to be unnecessary.

SUMMARY

The Michel ΔpH method was modified to provide a semi-micro method of determining rat whole blood cholinesterase activity.

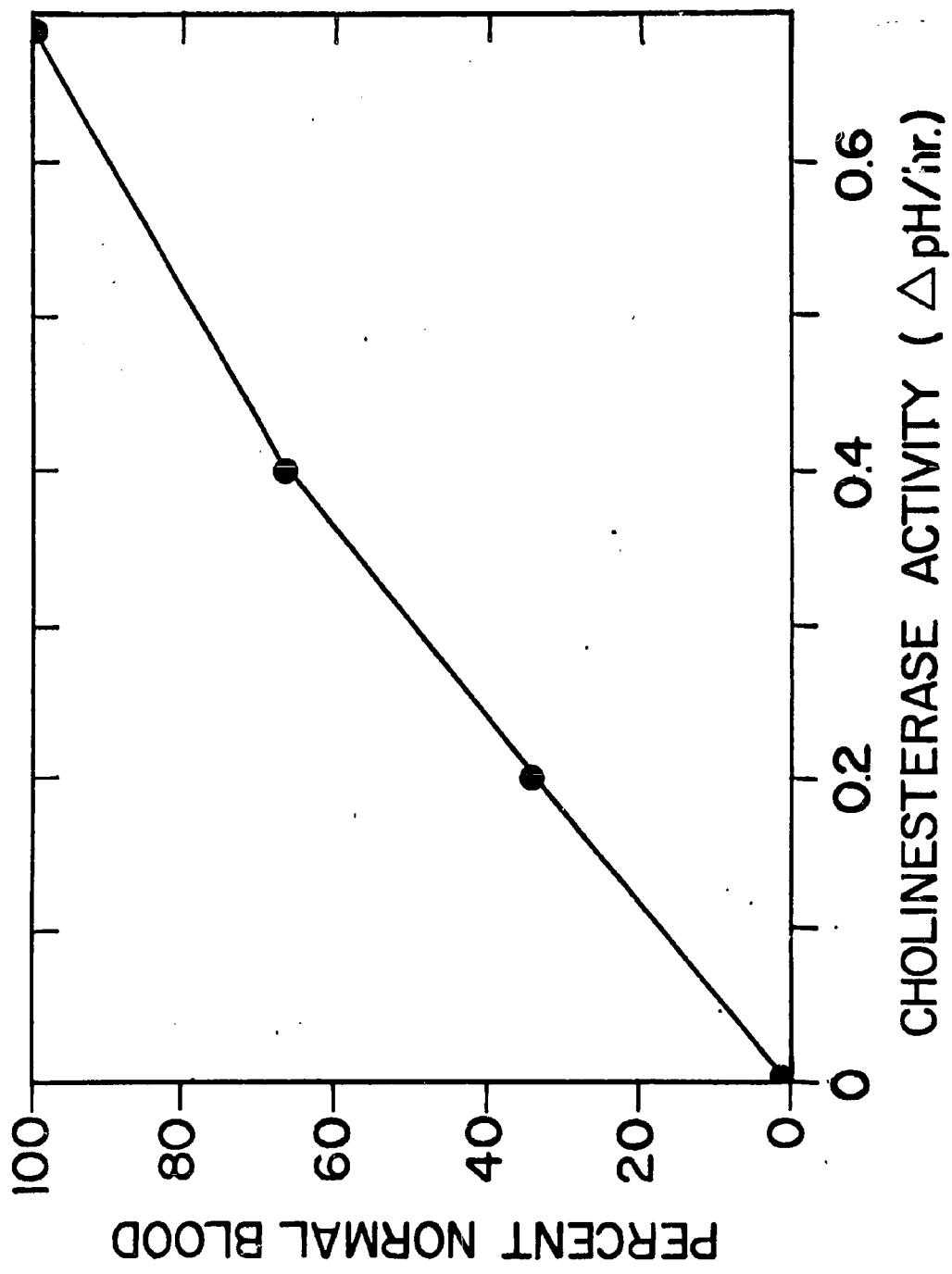
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SUMMARY

The Michel ΔpH method was modified to provide a semi-micro method of determining rat whole blood cholinesterase activity.

FIGURE I



PART III

STUDIES OF THE MECHANISMS OF TACHYPHYLAXIS IN ANIMALS

INTRODUCTION

Preliminary studies have been completed which indicate that tachyphylaxis can be produced in the experimental animal. Further studies to determine the mechanism of this phenomenon are underway.

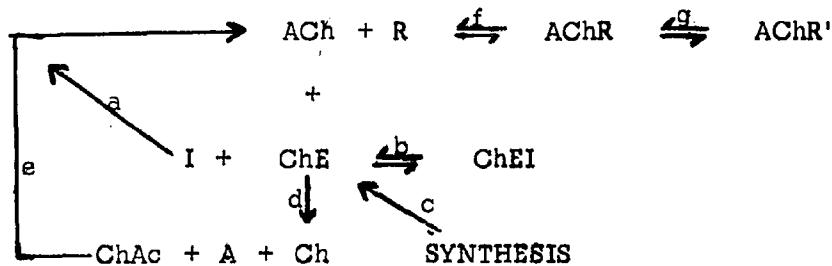


Figure 2

As far as is known, the lethal effect of anticholinesterase agents is due to the alteration of receptor produced by acetylcholine (reaction g, Figure 2). Tachyphylaxis or tolerance may be considered to be a reduction in the amount of this substance (AChR'). Possible mechanisms by which this might occur are shown diagrammatically in Figure 2, and may be enumerated as follows:

- a. increased destruction or sequestration of inhibitor (I)
- b. diminished reactivity between the inhibitor and cholinesterase (ChE)
- c. increased rate of cholinesterase synthesis
- d. diminished reactivity between cholinesterase and acetylcholine (ACh)
- e. diminished synthesis of acetylcholine by choline acetylase (ChAc)
- f. diminished reactivity between receptor (R) and acetylcholine
- g. diminished effect of acetylcholine on receptor (conversion to R')

Such an hypothetical formulation is of value, for it is amenable to experimental test. Possibilities a, b, and c imply that the inhibitor would produce less than the expected amount of cholinesterase inhibition. Possibility e would render the animal abnormally susceptible to the administration of choline acetylase inhibitor such as the hemicholinium, HC-3. Possibility f would render the animal insensitive to all depolarizing substances, including decamethonium. Thus, it should be possible to distinguish between these hypothetical

mechanisms by observing the effect of inhibitor on cholinesterase activity, and by comparing the susceptibility of the tolerant animal with the normal to the effect of hemicholinium, d-tubocurarine, and decamethonium.

PRELIMINARY STUDIES

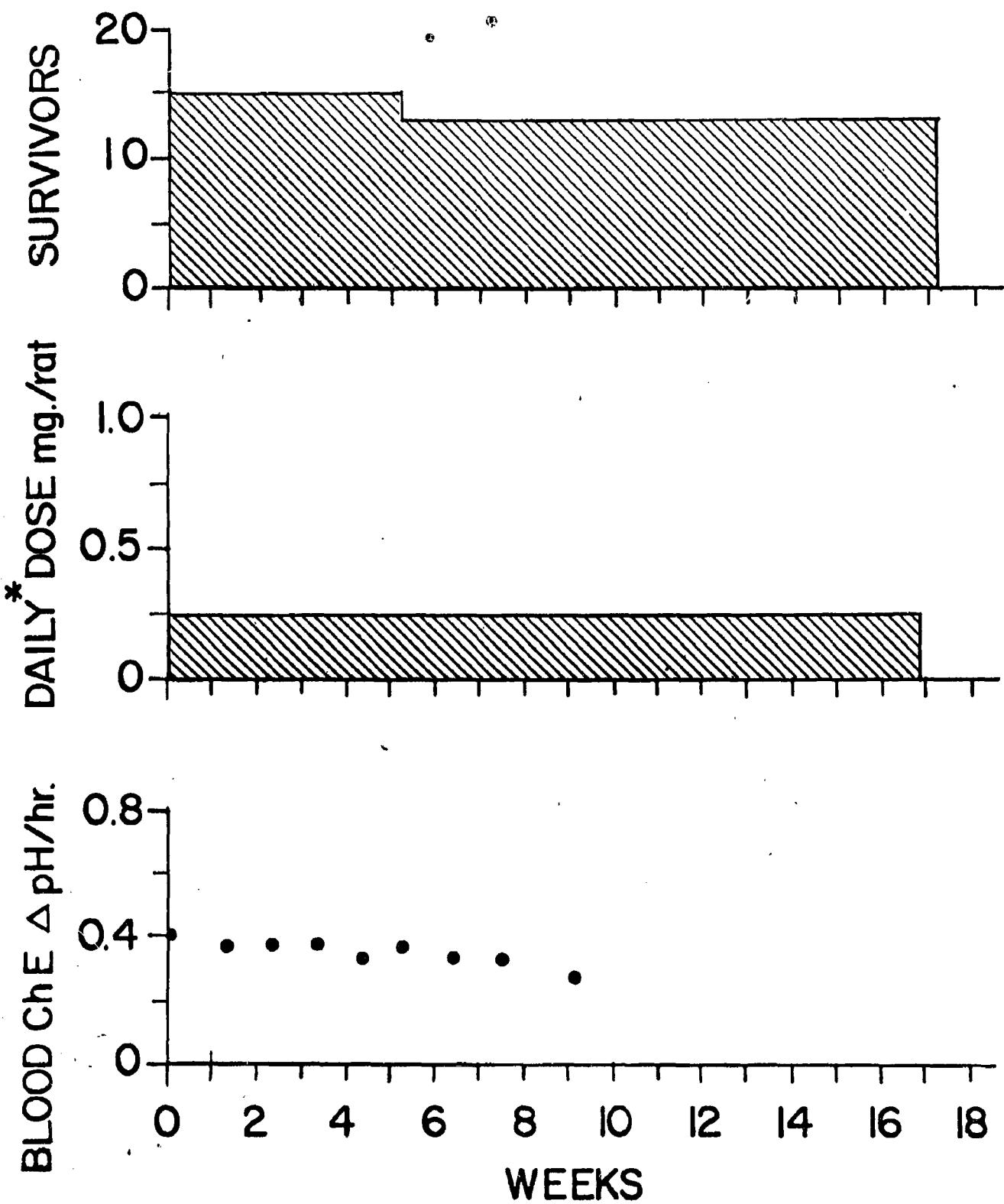
1. Materials and methods. Mestinon bromide 2 mg/ml was administered intraperitoneally to albino female rats of approximately 250 grams weight. Injections were made five days weekly in the doses shown in Figures 3 and 4. Whole blood cholinesterase was determined as described in Part II. Measurements were made both before and after injections of high doses of Mestinon and after the injection of low doses of Mestinon.

2. Results. Figure 3 summarizes the results on 15 rats administered Mestinon at the dose level of 0.25 mg/rat which is approximately equivalent to 1 mg/kg body weight. Since the acute LD-50 was determined to be 3.25 mg/kg body weight this dose represents 1/3 of the acute LD-50 five days weekly. The deaths which occurred in the sixth week were due to the inadvertent administration of 2/3 LD-50 to two of the rats. The cholinesterase determinations showed no tendency for the activity to rise after prolonged administration of the drug.

Data on the animals receiving progressively larger doses of Mestinon are summarized in Figure 4. The daily dose was administered five times weekly and was raised in a step fashion from 0.5 through 0.75 to 1.0 mg/rat. This latter dose is approximately equivalent to 4 mg/kg or 1 1/4 LD-50's. At this high level progressive mortality was experienced. The cholinesterase level did not vary significantly on these three dosage levels and there was evidence of persistent anticholinesterase effect in that the activity measured just prior to injection was subnormal.

3. Conclusions. It was demonstrated that tachyphylaxis to the lethal effect of the anticholinesterase drug Mestinon could be produced in animals. No evidence was found that this could be ascribed to increased destruction of inhibitor, decreased affinity between inhibitor and cholinesterase, or increased cholinesterase synthesis, for the effect on cholinesterase was not significantly reduced. These studies were handicapped by failure to obtain more than one control cholinesterase determination on the animals. Thus, in these experiments the failure to demonstrate significant difference in cholinesterase inhibition could be (1) due to the fact that there is no true difference or (2) due to the variation in cholinesterase between different individual rats obscuring a true difference.

In the group of animals which received high doses of Mestinon the other proposed studies were not done due to the deaths which occurred when the animals were receiving 1 1/4 LD-50's five days weekly. The experiment

FIGURE 3

must be so designed that there are no deaths among the tolerant animals, for results obtained on survivors are open to the interpretation that the more susceptible animals have thus been selected out of the population.

Detailed studies on the animals receiving the lower dose of Mestinon are presently underway.

e
ANALYSIS OF TACHYPHYLAXIS

1. Studies of neuromuscular transmission. One method of assessing the effect of d-tubocurarine, decamethonium, and hemicholinium is by quantitative measurement of its effect on neuromuscular transmission in control and tolerant rats. Methods were therefore devised to permit quantitative measurement and data were accumulated on the effect of these drugs on normal rats.

The methods we have previously employed in studying neuromuscular transmission in the cat were modified to permit application to the rat. Rats were anesthetized with chloral hydrate 400 mg/kg body weight intraperitoneally and artificial respiration was commenced through an endotracheal cannula. The gastrocnemius muscle was set up in an isometric myograph and supramaximal shocks were applied to the sciatic nerve. Single stimuli were applied every 10 seconds and tetanic responses were obtained with trains of stimuli at 20/second for 10 seconds. Tension was recorded from strain gages and was displayed on a Sanborn recorder. Care was taken to insure stability of the preparation. Drugs were injected intravenously via an exposed jugular.

Data from twenty-nine experiments were analyzed to determine the dose of drug which reduced twitch and tetanus to 50% of the control value (effective dose 50%, ED-50). These data are summarized in Table 3. The effective dose on twitch and tetanus were of the same order of magnitude for decamethonium and hemicholinium while d-tubocurarine had a much more pronounced effect on the tetanus. These observations are compatible with published results in other species. The time course of this effect on the twitch is documented in Table 4. As might be anticipated, increasing dose size had little effect on the onset of peak action, but increased the duration of action quite significantly. Detailed comparison between normal controls and the tolerant animals in the preliminary studies were not done. As was mentioned, the deaths occurring in the tolerant group would make any results difficult to interpret. This method of analysis is being used in studies currently underway.

2. Studies of lethality. As outlined in the introduction, the various hypotheses would predict differing effects on the lethality of d-tubocurarine, decamethonium, and hemicholinium. For this reason, the LD-50 of d-tubocurarine, decamethonium, and hemicholinium have been determined in normal rats. This will be compared with the effect of injecting an LD-50 of these drugs in tolerant animals. These studies are presently being performed on the rats in the preliminary study on low dose of Mestinon and

are projected on the animals currently being made tolerant.

* CURRENT STUDIES

At present, groups of rats are being injected with Mestinon 2 1/4 mg/kg which is equivalent to 3/4 LD-50. Another group is receiving DFP 0.24 mg/kg which is equivalent to 1/10 LD-50 twice weekly. Studies being performed on these animals include three control cholinesterase determinations to insure an accurate baseline followed by blood cholinesterase before and after a given dose of anticholinesterase. After five weeks of drug administration this effect on cholinesterase will be compared with that produced by a similar dose in parallel control animals. A portion of these animals will then receive 1 LD-50 of d-tubocurarine, decamethonium, or hemicholinium while others will receive an ED-50 of these drugs while recording neuromuscular transmission.

TABLE 3

DRUG	NO. OF EXPTS.	ED-50 (mg/kg i.v.) TWITCH	ED-50 (mg/kg i.v.) TETANUS
d-tubocurarine	14	0.15	<< 0.10
decamethonium	12	2 1/4	1 1/4
hemicholinium	13	3 1/4	2

TABLE 4

DRUG (i.v.)	DOSE (mg/kg)	TIME TO MAXIMAL EFFECT (min)			TIME TO 50% RECOVERY (min)		
		N	mean	range	N	mean	range
d-tubocurarine	0.10	7	6.5	2-15	4	38	20-60
	0.15	7	3.2	1-8	3	63	15-120
decamethonium	1.5	3	5.5	2-12	3	21	10-41
	2.0	6	7.9	1.5-20	3	149	
	2.5	2	13.5	10-17	1	148	
	3.0	1	9.5		1	>180	
hemicholinium	1.0	1	35		1	52	
	1.5	3	16.6	3-32	2	77	50-104
	2.0	5	6.5	1.5-15	3	38	15-80
	2.5	2	15		2	37	35-40
	3.0	1	4		1	>180	
	4.0	1	3		1	177	

PART IV

THE EFFECTS OF STEROIDS ON THE ACTION OF ANTICHOLINESTERASE AGENTS

INTRODUCTION

Adrenal cortical steroids have a striking effect on the defect in neuromuscular transmission in myasthenia gravis. During their period of administration there is an increase in the degree of block of neuromuscular transmission (following the cessation of steroid therapy there may or may not occur some improvement in the degree of block). Whatever the mechanism of this action, one would anticipate that any effect which might produce disruption of neuromuscular transmission in the myasthenic might produce improvement in neuromuscular transmission under circumstances of anticholinesterase intoxication.

PRELIMINARY RESULTS

Prednisone (1 mg per kg/day) and hydrocortisone (5 mg per kg/day) were administered in the food of 80 mice using the under-feeding technique. After 80 days of steroid administration prednisone, hydrocortisone, and control mice were administered an LD-50 of d-tubocurarine or decamethonium. Although there appeared to be increased lethality of both drugs in the steroid treated animals, these results were not statistically significant.

PRESENT STUDIES

Currently rats are being administered microcrystalline cortisone acetate intraperitoneally in the same dosage range and studies similar to those described for tachyphylaxis are planned.

PART V

MISCELLANEOUS STUDIES

Detailed studies of muscle electrolyte, membrane and end-plate potential under circumstances of chronic cholinesterase intoxication are in progress. The animals studied are those described in Part III. The techniques of analysis have been included in previous Annual Comprehensive Reports. Previous studies in vitro demonstrated that there was increased potassium efflux in the presence of anticholinesterase agent. These experiments were less than satisfactory because of abnormally high potassium efflux in the control specimens. This effect was presumably due to anoxia of the more central fibers during the period of incubation. The current in vivo approach to the problem appears to be simpler than use of brief in vitro incubation and a radioisotopic method of measuring ionic flux.

APPENDIX

Publications in the open literature resulting directly and indirectly from studies supported in part by Chemical Corps contract during the period 1951 - 1961.

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19. Grob, D., Johns, R.J., and Harvey, A.M. Studies in neuromuscular function; effects of anticholinesterase compounds, d-tubocurarine, and decamethonium in patients with myasthenia gravis. Bull. Johns Hopk. Hosp. 99, 219-238, 1956.

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